Six- to eight-week-old female Balb/c mice (Charles River Laboratories, Inc., Wilmington, MA) were injected intramuscularly (into the tibialis muscle) with 100 μg of pCWNVCp or pCDNA3 in phosphate buffered saline (PBS) and 0.25% bupivacaine-HCl (Sigma, St. Louis, MO). After 48 hr of transfection, the tibialis muscle was harvested. The fresh muscle tissue was then frozen in O.C.T. compound (Sakura Finetek USA, Inc., Torrance, CA). Four micron frozen sections were made using a Leica 1800 cryostat (Leica Inc., Deerfield, IL). To detect the presence of lymphocytes in muscle, the slides were stained with hematoxylin and eosin (H&E) stain (Vector Labs). The slides were viewed with a Nikon OPTIPHOT fluorescence microscope (Nikon Inc., Tokyo, Japan) using a 40X objective (Nikon Fluo 40X Ph3D2). Slide photographs were obtained using Nikon camera FX35DX with exposure control by Nikon UFX-II and Kodak Ektachrome 160T slide film. A dramatic infiltration of immune cells into the muscle of mice immunized with pCWNVCp is shown in Figure 16B.

The infiltrating cells were characterized by FACS analysis. The infiltrating cells were harvested from muscle by dissecting out the whole leg muscle and mincing with mechanical force as previously described in Kim *et al.*, 2000, Human Gene Therapy, *supra*. The cells were recovered by filtering them through a funnel with a glass wool plug. The infiltrating cells were identified by FACS using antibodies to CD4, CD8, Mac-3, CD11c, CD86, and B220 (Pharmingen) as previously described in Kim *et al.*, 2000, Human Gene Therapy, *supra* and Chattergoon *et al.*, 1990, J. Immunol., 160:5707-5718, which is incorporated herein by reference. Samples were analyzed using a Coulter EPICS®XL-MCL flow cytometer. The infiltrating cells from the mice immunized with pCWNVCp included T cells (both CD4+ and CD8+) and macrophages (detected with anti-Mac3 antibodies) (Figure 16C). The high levels of CD4+ and CD8+ T cells in the immunized muscle provides further evidence of a high level of T cell activation. On the other hand, the muscle section extracted from the mice immunized with pCDNA3 (control) did not show any sign of cellular infiltration. Taken together, these results demonstrate that antigen-specific immune responses can be efficiently generated via DNA vaccination.

Example 12: Alignment of WNV Capsid Protein with Other *Flavivirus* Capsid Proteins.

Figure 17 shows the alignment of WNV Cp protein with portions of capsid proteins from other *Flaviviruses*, including Kunjin virus (KJV), Japanese ecephalitis virus (JEV), and dengue virus (DEN2), indicating that there is a high degree of identity among these proteins.

Example 13: WNV Cp Protein Induces Apoptosis *In Vivo* and *In Vitro* Through the Mitochondrial Pathway.

The West Nile virus Cp protein, in the absence of other WNV gene products induces rapid nuclear condensation and cell death in tissue culture. Apoptosis is induced through the mitochondrial pathway, as the observed changes in mitochodrial membrane potential were accompanied by Caspase 9 activation and downstream Caspase 3 activation. Moreover, the apoptosis determinant domain was identified to reside in the 3' terminus of the WNV Cp protein by deletion mutation analysis. Following intramuscular injection of a WNV Cp expression cassette, apoptosis in muscle tissue was clearly observed. Most importantly, WNV Cp gene delivery into the striatum of mouse brain resulted in cell death through capsid induced apoptosis *in vivo*. These studies suggest that the capsid protein of the WNV is responsible for aspects of viral pathogenesis through induction of the apoptotic cascade, supporting the idea that inhibiting this apoptotic function can be exploited as a viable therapeutic approach for the treatment of WNV infection. Additionally, there is sequence identity/homology between the WNV capsid protein and a known apoptosis-inducing region of the HIV-1 *vpr* gene product (Ayyavoo *et al.*, 1997, Nat. Med., 3:1117-1123; Stewart *et al.*, 1997, J. Virol., 71:5579-5592, each of which is incorporated by reference) (Figs. 18 and 19).

Example 14: Comparison of WNV Cp and HIV Vpr with the Proteins of Other Apoptosis-Associated Viruses.

A Medline search for the terms "apoptosis," "encephalitis," and "meningitis"yielded a list of various viruses identified with such symptoms in infected individuals. The amino acid sequences of the proteins of these viruses were compared with the amino acid sequence for WNV capsid protein or HIV-1 89.6 Vpr protein.

Alignments with WNV capsid protein (Fig. 19)

1. HIV-1 - The WNV capsid protein and the HIV-1 Vpr, a known apoptosis-inducing protein, share sequence homology.

2. Herpes Simplex Virus (HSV) – Sequence alignment of the major capsid protein of the HSV with the WNV Cp indicated possible apoptotic inducing capabilities. Interestingly, destruction via encephalitis has been implicated to correlate with the outcome of the disease.

3. Ebola Virus is a member of the *Filovirus* genus within the *Filoviridae* family. This pathogen has been implicated with inducing hemorrhagic fever. The alignment of WNV capsid protein

and the Ebola nucleocapsid protein indicated detectable amino acid homology within the WNV and nef apoptosis domains. The glycoprotein alignment with the WNV capsid protein also displayed pro-apoptotic domain homology.

4. Rubella Virus is a member of the *Togaviridae* family, and has been implicated in inducing apoptosis from an *in vitro* standpoint. Sequence alignment of the Rubella virus capsid protein indicated homology with the WNV capsid protein, as well as with HIV-1 Vpr protein (see Fig 19), and Tat proteins (data not shown) within the apoptotic domains.

Alignment with HIV-1 89.6 Vpr (Fig. 19)

1. Sindbis Virus – Published data report the apoptotic nature of the Sindbis Virus, especially leading to neuronal cell death. Alignment of the p230 nonstructural protein of Sindbis Virus with HIV-1 Vpr protein (and with Tat protein (data not shown)), indicated isolated homology within the Bcl-2 associated apoptotic regions. Interestingly, recently published data implicated inhibition of Sindbis apoptosis via Bax.

2. Cucumber Mosaic Virus – Previously published reports have implicated cucumber mosaic virus in inducing profound cell killing by necrosis. However, recent data have indicated apoptotic characteristics associated with cell death within tomatoes. Interestingly, our sequence alignment with the vpr 89.6 with the CMV 2A protein also displayed apoptotic domain homology. Comparison with the Tat HIV gene also gave pro-apoptotic homology with the CMV capsid protein.

3. HTLV – Comparisons of this virus with the Tat protein of HIV-1 provided possible insights in apoptotic inducing capability of this virus. Sequence alignment of Tat with the HTLV-1 p27 protein exhibited sequence homology within an apoptotic domain.

4. Nipah Virus – This virus is a member of the *Paramyxoviridae* family and can be highly lethal in humans. A recent outbreak was observed in Singapore, thus increasing the possibilities of transference into the United States. In addition, the virus seems to have similar clinical outcomes to the West Nile Virus and to other viruses that target the cerebrospinal fluid and cause neural encephalitis. A comparison of the fusion protein of Nipah virus with HIV 89.6 Vpr protein gave an interesting correlation. Strong homology was seen in a cell cycle arrest domain within the Nipah fusion protein. This surface protein could be a strong DNA vaccine candidate; the implications are that it plays a crucial role in the development of apoptosis and cell cycle arrest.